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Hydroxytyrosol ameliorates oxidative stress and mitochondrial dysfunction in doxorubicin-induced cardiotoxicity in rats with breast cancer

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ABSTRACT

Oxidative stress is involved in several processes including cancer, aging and cardiovascular disease, and has been shown to potentiate the therapeutic effect of drugs such as doxorubicin. Doxorubicin causes significant cardiotoxicity characterized by marked increases in oxidative stress and mitochondrial dysfunction. Herein, we investigate whether doxorubicin-associated chronic cardiac toxicity can be ameliorated with the antioxidant hydroxytyrosol in rats with breast cancer. Thirty-six rats bearing breast tumors induced chemically were divided into 4 groups: control, hydroxytyrosol (0.5 mg/kg, 5 days/week), doxorubicin (1 mg/kg/week), and doxorubicin plus hydroxytyrosol. Cardiac disturbances at the cellular and mitochondrial level, mitochondrial electron transport chain complexes I–IV and apoptosis-inducing factor, and oxidative stress markers have been analyzed. Hydroxytyrosol improved the cardiac disturbances enhanced by doxorubicin by significantly reducing the percentage of altered mitochondria and oxidative damage. These results suggest that hydroxytyrosol improve the mitochondrial electron transport chain. This study demonstrates that hydroxytyrosol protect rat heart damage provoked by doxorubicin decreasing oxidative damage and mitochondrial alterations.

1. Introduction

Oxidative stress is a significant consequence in cardiac injury associated with doxorubicin (ADR) treatment [1]. Cardiac tissue is extremely susceptible to free radical-induced damage because of high aerobic metabolism, lesser amount of antioxidant defenses compared to other tissues [2], and the post-mitotic features of myocytes [3]. Marked hypotension, tachycardia, cardiac dilation ventricular failure, and higher activities of glutamate-oxaloacetic transaminase, lactate dehydrogenase, and creatinine phosphokinase enzymes are characteristics of ADR-caused cardiomyopathy [4]. At the ultrastructural level, myofibril loss, cytoplasmatic vacuolization, increased number of lysosomes, and mitochondrial dysfunction have all been reported [1,5]. These cytotoxic side effects of ADR have been attributed to several events: selective accumulation in the mitochondrial lipid membrane, redox cycling, and subsequent generation of reactive oxygen and nitrogen species (ROS and RNS) [1,6]. It has been shown that ADR redox cycling

Abbreviations: AASA, aminoadipic semialdehyde; ADR, doxorubicin; AIF, Apoptosis-inducing factor; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CEL, N^{ε}-(carboxyethyl)-lysine; CK, creatinine kinase; CML, N^{ε}-(carboxymethyl)-lysine; DBI, double bond index; GSA, glutamic semialdehyde; HT, hydroxytyrosol; i.v., intravenous; LDH, lactate d[1]ehydrogenase; MDAL, N^{ε}-malondialdehyde-lysine; METC, mitochondrial electron transport chain; NQO1, NAD(P)H quinine oxidoreductase-1; ROS, reactive oxygen species.

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takes place in the mitochondrial electron transport chain (METC) [7], more specifically at complex-I, which, alongside complex-III, are more substantial ROS generators in heart [8]. High mitochondrial ROS production after ADR administration results in molecular oxidative damage that affects membrane-bound proteins and enzymes, lipids, the mitochondrial genome, as well as significant other biomolecules [7,9].

The secoiridoid oleuropein, a phenolic compound found in virgin olive oil, has demonstrated protective effects against ADR toxicity, mainly due to its high antioxidant capacity [1] as other biomolecules [10]. Phenolic alcohol hydroxytyrosol (HT), another bioactive molecule found in olive oil, has highly similar antioxidant properties [11]. HT has other features such as iron chelative, anti-atherogenic, hypolipidemic, anti-inflammatory, anti-thrombotic, anti-microbial, and anti-tumor properties as well [11]. Moreover, HT is a good hypoglucemic [12] and anti-viral agent [13,14] that has demonstrated protective effects in rat cardiomyocytes in an ischemia–reperfusion model [15]. Our group has recently reported that HT (0.5 mg/kg) is able to suppress breast tumor growth in female Sprague-Dawley rats by modifying the expression of several tumor-related genes [16].

The present study addresses, for the first time, not only the potential protective role of HT against chronic cardiotoxicity generated by ADR in rats with breast cancer, but also the effects of sustained HT on oxidative stress at the cardiac level. To demonstrate this, authors measured cardiac abnormalities at the cellular and mitochondrial level through histopathology and electron microscopy. Total electron flow and the number of ROS-generating-sites into METC can potentially affect the rates of mitochondrial ROS production. Therefore, the content and activity of METC complexes I–IV were also examined. The steady-state levels of five markers of oxidative, glycoxidative, and lipoxidative damage to proteins were measured by gas chromatography/mass spectrometry. Since protein oxidation is secondarily influenced by the membrane' sensitivity to lipid peroxidation [17], the full fatty acid composition was also measured.

2. Materials and methods

2.1. Animals and reagents

Thirty-six female Sprague-Dawley rats (170 ± 20 g), were purchased from Harlan Interfauna Ibérica S.L (Barcelona, Spain) at 7 weeks of age. All animals were housed four per cage in an environmentally controlled room at 22 ± 2 °C with a 12:12 h (light/dark) cycle. They were given free access to rodent chow and deionized water. All experiments were performed in accordance with the principles of the Helsinki Declaration, Spanish animal welfare legislation and Ethical Committee of the University of Granada (CEEA 264-2008) (Spain). Unless otherwise specified, all reagents were from Sigma (Saint Louis, MO, USA). Hydroxytyrosol was purchased from Cayman Chemical (Ann Arbor, MI, USA). Doxorubicin was purchased from Pharmacia-Upjohn Laboratories, Bridgewater, NJ, USA.

2.2. Experimental protocol

Mammary tumors were induced as previously described [16]. Mammary tumor-bearing rats were randomized into four groups: (1) Control ($n = 10$): i.v. (intravenous) saline for 6 weeks; (2) HT ($n = 10$): HT (0.5 mg/kg, 5 days/week for 6 weeks); (3) ADR ($n = 8$): doxorubicin (i.v. 1 mg/kg/week for 6 weeks) for a total cumulative dose of 6 mg/kg; and (4) ADR + HT ($n = 8$): combo group treated with the same doses and timing than HT and ADR groups. One week after the last injection, animals were weighed, anaesthetized with intraperitoneally administered ketamine (Sigma, Saint Louis, MO, USA), and sacrificed by aortic bleeding. Whole blood was collected and plasma isolated. Hearts were immediately removed and weighed. One half of the heart was snap frozen in liquid nitrogen, while the other half was fixed in 4% buffered formalin (Sigma, Saint Louis, MO, USA). A small fragment was sectioned for electron microscopy.

2.3. Histopathological analysis

Formalin-fixed hearts were paraffin-embedded, sectioned (3 μm thickness) and placed onto glass slides. Briefly, paraffin-embedded tissue sections were deparaffinized with Neoclear (Panreac Quimica, Barcelona, Spain), rehydrated with graded alcohol, and stained with Harris' hematoxylin and eosin (Dako, Glostrup, Denmark) in a Leica Autostainer (Wetzlar, Germany). Billingham's grade [18] was established according to the following criteria: 0.0 = no lesions; 0.5 = abnormal heart but without typical hurt due to ADR-associated toxicity; 1.0 = ≤5%, 1.5 = 6–15%, 2.0 = 16–25%, 2.5 = 26–35%, 3.0 = >35% of the cells with proper lesions caused by ADR.

2.4. Electron microscopy analysis of cardiac mitochondria

Cardiac muscle samples were prefixed in 1.5% formaldehyde in 1% cacodylate buffer, pH 7.4 for 2 h at 4 °C and fixed in 1% osmium tetroxide for 60 min at 0–4 °C (Sigma, Saint Louis, MO, USA). Samples were dehydrated in graded ethanol and embedded in Epon resin and incubated overnight at 65 °C. Ultrathin sections (70 nm) were cut with a diamond knife using an ultrakut S ultramicrotome and placed on 200-mesh copper grids. All sections were stained with uranyl acetate, counterstained with lead citrate, and viewed using a Carl Zeiss (Oberkochen, Germany) EM10C electron microscope at 40,000× magnification in the Scientific Instrument Service, University of Granada. Negatives were digitally transformed into positive images. Mitochondrial area and percentage of altered mitochondria were assessed. Software Image J [19] was used for the quantification of mitochondrial parameters.

2.5. Biochemical parameters in plasma

Plasma levels of creatinine kinase (CK), lactate dehydrogenase (LDH), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were measured with enzymatic kits (Spinreact, Girona, Spain).

2.6. Immunoblot analysis of METC complexes I–IV and AIF

Mitochondrial complexes-I to -IV and AIF were estimated using Western-blot analysis as described previously [20]. Immunodetection was performed using specific antibodies (Molecular Probes, Invitrogen Ltd., UK): complex-I (39 kDa-NDUFA9 and 30 kDa-NDUFS3 subunits, 1:1000), complex-II (70 kDa-Flavoprotein subunit, 1:500), complex-III (48 kDa-CoreII and 29 kDa-Rieske iron–sulfur-protein subunits, 1:1000), complex-IV (57 kDa-COXI subunit, 1:1000), and AIF polyclonal antibody (1:1000). Relative intensity was determined using porin (1:5000, Molecular Probes, Invitrogen Ltd., UK) as control. Appropriate peroxidase-coupled secondary antibodies and chemiluminescence HRP (horse radish peroxidase) substrate (Millipore, MA, USA) were used for primary antibody detection. Signal quantification and recording was performed with ChemiDoc BioRad equipment (Bio-Rad Laboratories, Inc., Barcelona, Spain). Protein concentration was determined by the Bradford method.

2.7. Mitochondrial complexes-I and -IV activities

Complexes-I and -IV activities were determined using enzyme-activity-dipstick-assay kit from MitoSciences (MitoSciences Inc.